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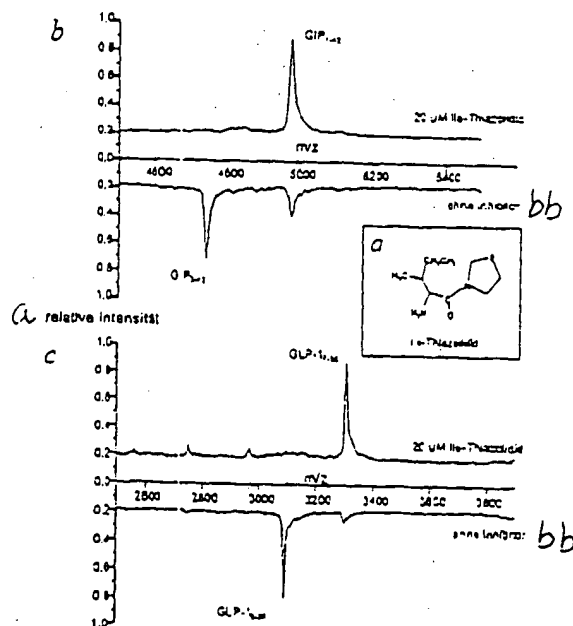
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54) Method for reducing the blood glucose level in mammals

57) Use of effectors of dipeptidyl peptidase (DP IV) and DP IV analog enzymatic activity for reducing the blood sugar level to below the glucose concentration characteristic for hyperglycemia in the serum of a mammalian organism.

Key to the Figure:

a - relative intensity; bb- without inhibitor;



Description

The invention describes a simple method for reducing the blood sugar concentration with the aid of activity-reducing effectors (substrates, pseudosubstrates, inhibitors, bonding proteins, antibodies, etc.) for enzymes with an activity comparable or identical to the enzymatic activity of the enzyme dipeptidyl peptidase IV.

In addition to processes which are included in nonspecific proteolysis, which, in the final analysis causes the degradation of proteins to amino acids, regulatory proteases are known which participate in the functionalization (activation, deactivation, modulation) of endogenous active peptide substances [KIRSCHKE, H., LANGNER, J., RIEMANN, S., WIEDERANDERS, B., ANSORGE, S. and BOHLEY, P., Lysosomal cysteine proteases. *Excerpta Medica (Ciba Foundation Symposium 75)*, 15 (1980); KRÄUSSLICH, H.-G. and WIMMER, E., Viral Proteinases, *Ann. Rev. Biochem.*, 57, 701 (1987)]. Especially, in connection with immune research and neuropeptide research, a number of such so-called convertases, signal peptidases or enkephalinases have been discovered [GOMEZ, S., GLUSCHANKOF, P., LEPAGE, A., MARRAKCHI, N. and COHEN, P., *Proc. Natl. Acad. Sci. USA* 85, 5468 (1988); ANSORGE, S. and SCHÖN, E., *Histochem.* 82, 41 (1987)].

Based on the frequency of occurrence of the amino acid proline in a number of peptide hormones and the related structural properties of these peptides, a signal-peptidase-analog function was discussed for proline-specific peptidases [YARON, A., The Role of Proline in the Proteolytic Regulation of Biologically Active Peptides. *Biopolymers* 26, 215 (1987); WALTER, R., SIMMONS, W. H. and YOSHIMOTO, T., Proline Specific Endo- and Exopeptidases. *Mol. Cell. Biochem.* 30, 111 (1980); VANHOOF, G., GOOSSENS, F., L. DE MEESTER, D. HENDRIKS and S. SCHARPE, Proline motifs and their biological processing. *FASEB Journal* 9, 736 (1995)]. Due to its special structure, as well as conformation, in these peptides, proline provides the stability of these peptides by protecting them against degradation by nonspecific proteases [KESSLER, H., Conformation and Biological Activity of Cyclic Peptides. *Angew. Chem.* 94, 509 (1982)]. On the other hand, enzymes which act highly specifically, changing the structure of proline-containing sequences (HIV

proteases, cyclophyllin, etc.) are attractive goals of present active agent research. Especially, for peptidases which cleave after proline, prolyl endopeptidase (PEP) and dipeptidyl peptidase IV (DP IV), relationships between the modulation of biological activity of natural peptide substrates and their selective cleavage by these enzymes were shown to be likely. Thus, it is assumed that PEP plays a role in the learning and in the cognitive process and DP IV is involved in signal transfer during immune response [S. ISHIURA, T. TSUKAHARA, T. TABIRA, T. SHIMIZU, K. ARAHATA and H. SUGITA, FEBS Letters 260, 131 (1990); M. HEGEN, G. NIEDOBITEK, C. E. KLEIN, H. STEIN AND B. J. FLEISCHER of Immunology 144, 2908 (1990)].

Similarly to the extraordinary proline-specificity of these enzymes, their high selectivity for the amino acid alanine within typical recognition regions in substrates of these enzymes are discussed, according to which alanine-containing peptides can assume conformations similar to the proline-containing peptides with analogous structure. Recently, such properties of alanine-containing peptide chains were detected by point mutation (exchange of proline against alanine) [R. W. DODGE and H. A. SCHERAGA, Folding and unfolding kinetics of the proline-to-alanine mutants of bovine pancreatic ribonuclease A. Biochemistry 35 (5) 1548 (1996)].

DP IV or DP IV-analog activity (for example, the cytosolic DP II has a substrate specificity which is almost identical to that of DP IV) occurs in the blood circulation, where it cleaves highly specific dipeptides from the N-terminus of biologically active peptides when proline or alanine are the neighboring residues of the N-terminal amino acid in their sequence. Therefore, it is assumed that this enzyme is involved in the regulation of polypeptides in vivo [G. VANHOFF, F. GOOSSENS, L. DE MEESTER, D. HENDRIKS and S. SCHARPE, Proline motifs and their biological processing. FASEB Journal 9, 736 (1995)].

The glucose-dependent insulinotropic polypeptides: Gastric Inhibitory Polypeptide I-42 (GIP₁₋₄₂) and Glucagon-Like Peptide Amide-1 7-36 (GLP-1₇₋₃₆), hormones, which stimulate the glucose-induced insulin secretion of the pancreas (also integrins), are substrates of DP IV, because it can cleave off the dipeptides tyrosinyl-alanine and histidyl-alanine from the N-terminal sequences of these peptides in vitro and in situ [R. MENTLEIN, B.

GALLWITZ and W. E. SCHMIDT, Dipeptidyl Peptidase IV hydrolyzes gastric inhibitory polypeptide, glucagon-like peptide-1(7-36)amide, peptide histidine methionine and is responsible for their degradation in human serum. *Eur. J. Biochem.* 214, 829 (1993)].

The reduction of such DP IV and DP IV-analog enzyme activity for the cleavage of such substrates in vivo can be used to suppress effectively undesired enzyme activity under laboratory conditions as well as in pathological states of mammalian organisms [H.-U. DEMUTH, Recent developments in the irreversible inhibition of serine and cysteine proteases, *J. Enzyme Inhibition* 3, 249-278 (1990); H.-U. DEMUTH and J. HEINS, On the catalytic Mechanism of Dipeptidyl Peptidase IV, in *Dipeptidyl Peptidase IV (CD 26) in Metabolism and the Immune Response* (B. Fleischer, Ed.) R. G. Landes, Biomedical Publishers, Georgetown, 1-35 (1995)]. For example, Diabetes mellitus type II (also called adult-onset diabetes) is based on reduced insulin secretion or disturbances in the receptor function, which are caused among others by proteolytically produced concentration anomalies of integrins [J. C. BROWN, M. DAHL, S. KWAWK, C. H. S. MCINTOSH, S. C. OTTE and R. A. PEDERSON, *Peptides* 2, 241 (1981); W. E. SCHMIDT, E. G. SIEGEL, B. GALLWITZ, H. KUMMEL, R. EBERT and W. CREUTZFELDT, Characterization of the inulinotropic activity of fragments derived from gastric inhibitory polypeptide. *Diabetologia* 29, 591A (1986); K. ADELHORST, B. B. HEDEGAARD, L. B. KNUDSEN and O. KIRK, Structure-activity studies of glucagon-like peptide. *J. Biol. Chem.*, 269, 6275 (1994)].

Hyperglycemia and the related causes and sequelae (also Diabetes mellitus) are treated according to the present state of the art by the administration of insulin (for example, isolated from bovine pancreas and also material obtained by genetic engineering) to diseased organisms in various forms of administration. All the more modern methods that have become known so far are characterized by high material expenditure, high cost and frequently by a decisive adverse effect on the quality of life of the patients. The classical method (daily iv insulin injection, usually starting at the age of 30) treats the acute disease symptoms, but, after long application, leads to severe alterations of the vessels (arteriosclerosis) and nerve damage [P. LACY, Status of Islet Cell Transplantation. *Diabetes Care* 16 (3), 76 (1993)].

Recently, the installation of subcutaneous depot implantates (the released insulin is dosed so that the daily injections can be omitted) as well as implantation (transplantation) of intact Langerhans cells into the pancreas gland, the function of which is disturbed or other organs and tissues, have been proposed. Such transplantations are technically expensive. Furthermore, they represent a surgical intervention in the receiving organism, which involves risks and also require, even in cell transplantations, methods for suppression or by-passing the immune system [P. LACY, Treating Diabetes with Transplanted Cells. *Sci. Americ.* 273 (1) 40-46 (1995)].

On the other hand, the possible oral application of high affinity, low molecular enzyme inhibitors is a more cost-effective alternative, for example, than invasive surgical techniques in the treatment of pathological phenomena. Such enzyme inhibitors have found in the meantime therapeutic application as immunosuppressants, antithrombotic as well as AIDS virostatic agents. With the aid of chemical design of stability, transport and clearance properties, the mode of action can be modified and adapted to individual properties [M. SANDLER and H. J. SMITH, Editors, *Design of Enzyme Inhibitors as Drugs*, Oxford University Press, Oxford (1989); J. E. MUNROE, T. A. SHEPHERD, L. N. JUNGHEIM, W. J. HORNBACH, S. D. HATCH, M. A. MUESING, M. A. WISKERCHEN, K. S. SU, K. M. CAMPANALE, A. J. BAXTER and J. M. COLACINO, Potent, orally bioavailable HIV-1 protease inhibitors containing noncoded D-amino acids. *Bioorg. Medicinal Chem. Letters* 5 (23), 2897 (1995)].

The goal of the invention is a simple and novel method for reducing the blood-glucose level, which can be achieved according to the invention by the fact that, upon administering effectors to a mammalian organism, in causal sequence, the endogenously (or additionally exogenously) administered insulintropic peptides GIP_{1-42} and GLP-1_{7-36} (among others GLP-1_{7-37} or its analogs) are degraded less by DP IV or DP IV-like enzymes and thus the concentration decrease of these peptide hormones or their analogs is reduced or delayed.

Surprisingly, the invention is based on the finding that a reduction of DP IV or DP IV-like enzymatic activity acting in the blood circulation can lead causally to influencing the blood sugar level. It was found that

1. the reduction of DP IV or DP IV-analog activity leads to a relative increase in the stability of glucose-stimulated or externally introduced integrins (or of their analogs), that is, by application of effectors of DP IV or DP IV-analog proteins, one can control the degradation of integrins in the blood.
2. increased stability of integrins (or of their analogs) to biodegradation results in a change of activity of endogenous insulin.
3. the increase in stability of the integrins in the blood achieved by reduction of DP IV or DP IV-analog enzymatic activity leads to subsequent alteration of the glucose-induced insulin action and thus to a modulation of the blood glucose level which can be controlled by DP IV effectors.

The effectors of DP IV or DP IV-analog enzymes applied according to the invention can be used in pharmaceutically applicable formulation complexes as inhibitors, substrates, pseudosubstrates, inhibitors of DP IV expression, bonding proteins or antibodies of these enzyme proteins or combinations of these different substances which reduce the DP IV or DP IV-analog protein concentration in the mammalian organism. The effectors according to the invention are DP IV inhibitors, such as the dipeptide derivatives or dipeptide mimetics, alanyl pyrrolidide, isoleucyl thiazolidide, as well as the pseudosubstrate N-valyl prolyl, O-benzoyl hydroxylamine. Such compounds are known from the literature [H.-U. DEMUTH, Recent developments in the irreversible inhibition of serine and cysteine proteases, J. Enzyme Inhibition 3, 249 (1990)] or can be prepared in analogy to methods described in the literature.

The method according to the invention represents a novel procedure for reducing elevated blood glucose concentration in the serum of mammals. It is simple, commercially useful and is suitable for application in therapy in human medicine, especially of diseases which are based on excessive blood glucose values.

The effectors are administered in the form of a pharmaceutical preparation containing the active ingredient in combination with the usual carrier materials known in the state of the

art. For example, they are applied parenterally (for example, iv in physiological sodium chloride solution) or enterally (for example, orally, formulated with the usual carrier materials, for example, glucose).

Depending on their endogenous stability and their bioavailability, single or even multiple administrations of the effectors must occur in order to achieve the desired normalization of the blood glucose values. For example, in the case of aminoacyl thiazolidides, such dosage range lies between 1.0 mg and 10.0 mg effector substance per kilogram.

Practical Examples

Example 1. Inhibition of DP IV-catalyzed hydrolysis of the integrins GIP_{1-42} and GLP-1_{7-36} in situ

The hydrolysis of integrins caused by DP IV or DP IV-analog activity can be detected in vitro with purified enzyme as well as in situ, for example, in pooled human serum, or can be suppressed with the aid of inhibitors (Figure 1).

According to the invention, in situ, by incubation of $30\ \mu\text{M}$ GIP_{1-42} or $30\ \mu\text{M}$ GLP-1_{7-36} and $20\ \mu\text{M}$ isoleucyl thiazolidide (1a), of a reversible DP IV inhibitor in 20% serum at pH 7.6 and 30°C , complete suppression of the enzyme-catalyzed hydrolysis of both peptide hormones can be reached within 24 hours (1b and 1c, both the upper spectra). Synthetic GIP_{1-42} ($5\ \mu\text{M}$) and synthetic GLP-1_{7-36} ($15\ \mu\text{M}$) were incubated with human serum (20%) in 0.1 mM TRICINE buffer at pH 7.6 and 30°C for 24 hours. Samples of the incubation batches (for GIP_{1-42} $2.5\ \mu\text{mole}$ and in the case of GLP-1_{7-36} $7.5\ \mu\text{mole}$) were taken after various time intervals. The samples were co-crystallized with 2',6'-dihydroxyacetophenone as matrix and analyzed with MALDI-TOF mass spectrometry. The spectra (Figure 1) represent accumulations of 250 individual laser shots per sample.

(1b) The signal in the range of $m/z\ 4980.1 \pm 5.3$ corresponds to GIP_{1-42} (M4975.6) and $m/z\ 4745.2 \pm 5.5$ to the DP IV hydrolysis product GIP_{3-42} (M4740.4).

(1c) The signals m/z 3325.0 ± 1.2 correspond to GLP-1₇₋₃₆ (M3297.7) and m/z 3116.7 ± 1.3 to the DP IV hydrolysis product GLP-1₉₋₃₆ (M3089.6).

In the experimental batches without inhibitor, the integrins were completely degraded during this time period (Figure 1b, 1c, both lower spectra).

**Example 2. Inhibition of the degradation of GLP1 42
by the DP IV inhibitor isoleucyl thiazolidide in vivo**

If one follows the metabolism of the native integrins (here GLP-1₇₋₃₆) in the serum of rats as a function of the presence of the DP IV inhibitor, isoleucyl thiazolidide (iv injection of a 1.5 μ M inhibitor solution in 0.9% sodium chloride solution) in comparison to a control, then, at a concentration of the isoleucyl thiazolidide inhibitor of approximately 0.1 mg/kg of laboratory rats, in the inhibitor-treated experimental animals ($n = 5$) no degradation of the insulinotropic peptide hormone GLP-1₇₋₃₆ is observed during the experimental period (Figure 2).

For the detection of the metabolites in the presence and in the absence of the DP IV inhibitor (20 minutes after prior administration of iv inhibitor or sodium chloride), the experimental and control animals received iv 50-100 pM of 125 I-GLP-1₇₋₃₆ (specific activity approximately 1 μ MCi/pM). Blood samples were taken after 2-5 minutes and the plasma was extracted with 20% acetonitrile. Then the peptide extract was separated with RP-HPLC and the radioactivity of the fractions was analyzed in a γ -counter. The found activity is given in cpm (counts per minute relative to the maximum).

**Example 3. Modulation of the insulin action and reduction of the blood glucose level
after iv application of the DP IV inhibitor isoleucyl thiazolidide in vivo**

In rats stimulated by intraduodenal (id) injection of glucose, a time-delayed reduction of the glucose level can be observed after iv administration of different DP IV effectors, for example, of 0.1 mg of isoleucyl thiazolidide per kg of rat, due to the inhibitory action. This effect is dose-dependent and reversible after the infusion of

0.05 mg/min of the DP IV inhibitor isoleucyl thiazolidide per kg of rat is discontinued. The iv application of the same amount of glucose in inhibitor-treated and control animals shows no comparable action, in contrast to the experimental animals stimulated by id administration of glucose.

Figure 3 shows the relationship of the inhibitor-dependent changes of the plasma parameters: A - DP IV activity, B - plasma insulin level, C - blood glucose level.

The experimental animals ($n = 5$, male Wistar rats, 200-225 g) received as initial dose 1.5 μM of isoleucyl thiazolidide in 0.9% sodium chloride solution (\blacktriangle) or the same volume of 0.9% sodium chloride solution without inhibitor (\blacksquare) (control group $n = 5$). Furthermore, the experimental group received an infusion of the inhibitor at 0.75 $\mu\text{M}/\text{min}$ over a 30 minute experimental period (*). During the same time period, the control group was infused an inhibitor-free 0.9% sodium chloride solution. At the time $t = 0$, the animals received a glucose dose of 1 g/kg of 40% dextrose solution (w/v) intraduodenally.

Blood samples were taken at 10-minute intervals from all experimental animals.

Glucose measurements were made in whole blood (Lifescan One Touch II analyzer), while the DP IV activity and the insulin concentrations were determined in the plasma.

The insulin test used here is sensitive between 10 and 160 mU/mL [R. A. PEDERSON, A. M. J. BUCHAN, S. ZAHEDI-ASH, C. B. CHEN and J. C. BROWN, *Reg. Peptides*, 3, 53-63 (1982)]. The DP IV activity was determined spectrophotometrically [H.-U. DEMUTH and J. HEINS, *On the catalytic Mechanism of Dipeptidyl Peptidase IV; in Dipeptidyl Peptidase IV (CD 26) in Metabolism and the Immune Response* (B. Fleischer, Ed.) R. G. Landes Biomedical Publishers, Georgetown, I-35 (1995)]. All measured values are given as mean values with standard deviation.

Patent Claims

1. Application of effectors of dipeptidyl peptidase (DP IV) or of DP IV-analog enzyme activity to reduce the blood sugar level to below the glucose concentration characteristic for hyperglycemia in the serum of a mammalian organism.
2. Application according to Claim 1, characterized by the fact that the administration of effectors of the DP IV or DP IV-analog enzyme activity in mammals serves to prevent or ameliorate pathological metabolic anomalies of mammalian organisms, selected from glucosuria, hyperlipidemia, metabolic acidosis and Diabetes mellitus.
3. Application according to Claim 1, characterized by the fact that inhibitors, substrates, pseudosubstrates, inhibitors of DP IV expression, bonding proteins or antibodies of these enzyme proteins or combinations of the above effectors are used as effectors of dipeptidyl peptidase (DP IV) or DP IV-analog enzyme activity.

3 Page(s) of drawings attached
